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Isothiazoles as active-site inhibitors of HCV NS5B polymerase

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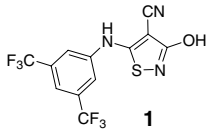
Abstract—Isothiazole analogs were discovered as a novel class of active-site inhibitors of HCV NS5B polymerase. The best compound has an IC_{50} of 200 nM and EC_{50} of 100 nM, which is a significant improvement over the starting inhibitor (**1**). The X-ray complex structure of **1** with HCV NS5B was obtained at a resolution of 2.2 Å, revealing that the inhibitor is covalently linked with Cys 366 of the '*primer-grip*'. Furthermore, it makes considerable contacts with the C-terminus, β-loop, and more importantly, to the active-site of the enzyme. The uniqueness of this binding mode offers a new insight for the rational design of novel inhibitors for HCV NS5B polymerase.

Infection by hepatitis C virus (HCV) is a global health crisis. An estimated more than 170 million individuals worldwide have been chronically infected, with 3–4 million new infections believed to occur each year.[1] Many infected individuals will develop liver damage with an increased risk of progression to fibrosis, cirrhosis, and liver cancer.[2] The current standard HCV therapy approved by FDA shows a sustained viral response for about half of the patients.[3] Hence, there is an increasing demand for new anti-HCV therapies to fulfill this unmet medical need.

NS5B polymerase is one of the virus-encoded proteins in the HCV genome and has been demonstrated to be essential for viral replication and transcription.[4] Medicinal chemistry pursuits of this polymerase for anti-HCV drug-discovery have led to the identification of numerous classes of structurally diversified inhibitors.[5–13] One of the reported NS5B inhibitors, NM283, is currently in phase II clinical trials,[13] thus validating NS5B as a target for the discovery of anti-HCV drugs.

We have previously described a series of allosteric inhibitors for HCV NS5B.[5,6] Our continued endeavors were focused on finding other mechanistically unique scaffolds. One such effort resulted in the discovery of compound **1** which was subsequently found to be a direct active-site inhibitor. This compound has an IC_{50} of 5.9 μM against BK strain of NS5B. Furthermore, it has an EC_{50} of 2.0 μM in a cell-based genotype 1b replicon system, and a CC_{50} of greater than 100 μM. Compound **1** thus provides us with a good lead for optimization due to its small molecular weight, reasonable enzymatic and replicon potencies, as well as its minimal cell toxicity. Herein, we describe our design, synthesis, and SAR of isothiazole derivatives as HCV NS5B polymerase inhibitors, together with an X-ray crystallographic structural study.



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The desired compounds were synthesized according to Scheme 1. In general, anilines (**2**) were obtained from commercial sources or prepared by reduction of the nitro compounds. Reaction of aniline (**2**) with thiophosgene afforded isothiocyanate (**3**), which was then reacted with 2-cyanoacetimide to form thioamides (**4**), which were oxidized to the desired isothiazoles (**5**).

Intermediate **7**, the key reagent for the synthesis of the mono-substituted sulfonamide of analog **5**, was prepared in three steps from **6** (Scheme 2). Once the aniline

Keywords: HCV; NS5B; NS5B inhibitor; HCV NS5B polymerase; HCV NS5B inhibitors; Isothiazole; NS5B active-site inhibitor; Covalent inhibitor.
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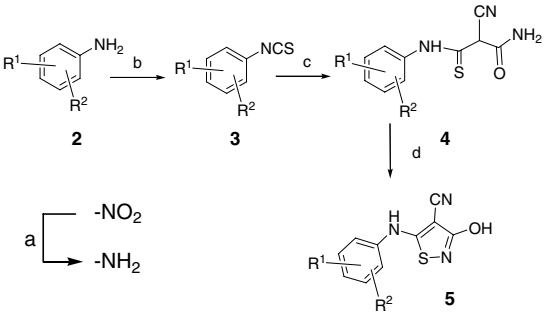
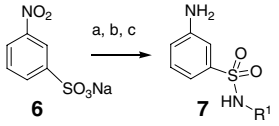


Scheme 1. Reagents and conditions: (a) Fe/NH_4Cl, EtOH(aq, 70%), 90 °C, 2 h, 70–90%; (b) $CSCl_2$, $CHCl_3$, rt, 2 h, 70–95%; (c) 2-cyanoacetimide, DMF, KOH, rt, 16–40 h, 65–87%, (d) Br_2, EtOAc, rt, 2.5 h, 70–90%.

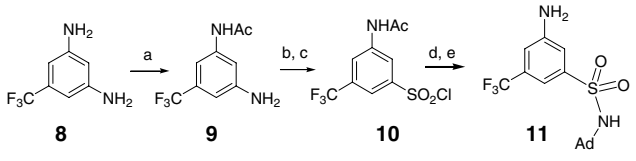


Scheme 2. Reagents and conditions: (a) $SOCl_2$, DMF, 0 °C → rt, 3 h, 19%; (b) R_1–NH_2, TEA, DMAP, THF, rt, overnight, 40%; (c) Fe/NH_4Cl, EtOH(aq, 70%), 90 °C, 3 h, 80%.

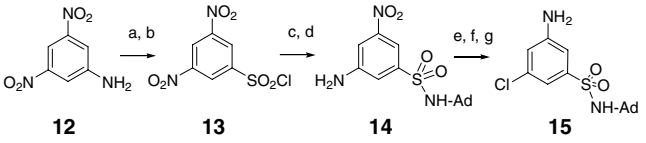
(**7**) was acquired, it was readily converted to the desired analog **5** according to Scheme 1.

For $R^1 = CF_3$, and $R^2 = SO_2NH$-adamantane (−Ad), the related analog **5** was synthesized through an intermediate **11** (Scheme 3). Commercially available diamine (**8**) was acylated to a mono-amine (**9**), which was further converted into sulfonyl chloride (**10**) via diazotization according to an established procedure.[14,15] Coupling of **10** with Ad-NH_2, followed by de-protection, yielded the desired intermediate (**11**), which was ultimately converted into the corresponding analog **5** using procedures described in Scheme 1.

The aniline intermediate **15**, required for the synthesis of analog **5** with R^1 = Cl, and $R^2 = SO_2NH$-Ad, was generated according to Scheme 4. Di-nitro-aniline **12** was converted into sulfonyl chloride **13**,[14,15] followed by coupling prior to reduction of one of the two nitro groups by $(NH_4)_2S$,[16] to give **14**. Another diazotization to convert –NH_2 to –Cl, followed by reduction, afforded **15**, which set the stage to synthesize the related analog **5** following the procedures in Scheme 1.



Scheme 3. Reagents and conditions: (a) AcCl, pyridine, rt, 24 h, 33%; (b) $NaNO_2$, HCl, HOAc, 0 °C, 30 min; (c) SO_2(gas), CuCl(I), HOAc, HCl, 0 °C → rt, 20 min, 58%; (d) R–NH_2, pyridine, THF, DMAP(cat.), rt, 16 h, 82%; (e) i—NaOH(aq)/dioxane, 80 °C, 3 h; ii—HCl(aq, 1 N), rt, 79%.



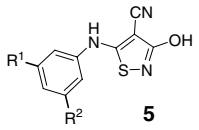
Scheme 4. Reagents and conditions: (a) $NaNO_2$, HCl, HOAc, 0 °C, 25 min; (b) SO_2(gas), CuCl(I), HOAc, HCl, 0 °C → rt, 40 min 56%; (c) Ad-NH_2, pyridine, THF, DMAP(cat.), rt, 16 h, 67%; (d) $(NH_4)_2S$, EtOH, reflux, 1 h, 84%; (e) same as (a); (f) CuCl, HCl, 60 °C, 1 h, 72%; (g) Fe/NH_4Cl, EtOH(aq, 70%), 92 °C, 1 h, 84%.

The compounds prepared above were then evaluated against a recombinant HCV NS5B polymerase using the enzymatic assay described previously.[17] The IC_{50} values are shown in Tables 1–3.

As shown in Table 1, all of the compounds bearing electron-withdrawing group(s) on the phenyl ring have IC_{50} values in the nano- to low micro-molar range. Replacement of $–CF_3$ by an electron-donating group eliminated the activity. For example, when R^1 and/or R^2 were replaced by Me, or –OMe, the resulting compounds, **16**–**18**, were inactive with IC_{50} values greater than 100 μM, indicating the importance of the electron-withdrawal groups. Introduction of a slightly less electron-withdrawing, but a slightly bulkier, group (**22** and **23**), resulted in about 3–4-fold boost of the IC_{50}, probably because of the stronger hydrogen-bonding ability of $–CO_2R$ than $–CF_3$. Interestingly, when CF_3 (**1**) was substituted with F or Cl, the resulting compounds (**19**–**21**) demonstrated significantly enhanced potencies. This is reflected by the observations that IC_{50} values of **19**–**21** are all in the nano-molar range. Particularly, **20** and **21** achieved IC_{50} values of 300 and 200 nM, respectively, which are nearly 20–30-fold more potent than the original compound (**1**) (5.9 μM). Such an improvement perhaps originates from a combined effect of bulkiness and electron-withdrawing capability. F and Cl atoms are less bulky than CF_3, and they are also slightly less electron-withdrawing as revealed by their smaller values of Hammett constant (σ)—a parameter measuring the electron-withdrawing ability.[18] For example, F or Cl has similar σ to $–CO_2R$, +0.34 (F), +0.37 (Cl) vs +0.36 (CO_2Me), but less than $–CF_3$ (+0.43).

Intrigued by the substantial outcome of electron-withdrawing groups on the potency, we decided to design and synthesize compounds containing substituents with greater σ values than CF_3. Mono-substituted compounds carrying $–SO_2NH_2$ (**24**), $–SO_2Me$ (**25**), or $–SO_2Bu$ (**26**), all with $\sigma \sim +0.60$, are slightly weaker inhibitors, with IC_{50} values of 9.9, 9.7 and 8.3 μM, respectively. Compound **27**, similarly mono-substituted, but further linked with a more bulky adamantane (Ad) group, had a potency increase of 7-fold versus **24** and 4-fold versus **1**. The improved IC_{50} of **27** indicates a potentially favorable hydrophobic interaction between the Ad group and the enzyme. Moreover, modeling suggested that this same Ad group overlaps in part with an inhibitor described by Pfefferkorn et al.,[10,19] prompting us to theorize that its combination with CF_3 or Cl of **1** or **21** might offer superior IC_{50}s. As a

Table 1. IC_{50} values of 3,5-substituted-isothiazole derivatives **5** (in μM)



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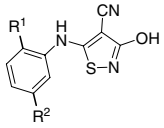
Compound	R^1	R^2	IC_{50}	Compound	R^1	R^2	IC_{50}
1	CF_3	CF_3	5.9	**23**	CO_2H	CF_3	1.5
16	Me	H	100	**24**	SO_2NH_2	H	9.7
17	Me	Me	100	**25**	SO_2Me	H	9.7
18	OMe	H	100	**26**	SO_2Bu	H	8.3
19	H	F	0.9	**27**	SO_2NH-Ad	H	1.4
20	F	F	0.3	**28**	SO_2NH-Ad	CF_3	3.5
21	Cl	Cl	0.2	**29**	SO_2NH-Ad	Cl	1.8
22	CO_2Me	CF_3	2.0	**30**	CF_3	Br	0.3

Table 2. IC_{50} values of 2,5-substituted-isothiazoloes (μM)



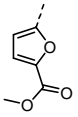
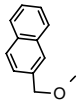
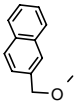
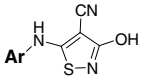
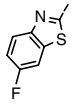
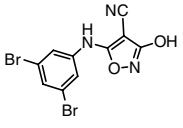
Compound	R^1	R^2	IC_{50}	Compound	R^1	R^2	IC_{50}
31		H	1.3	**35**		Br	0.3
32		F	0.5	**36**	–CO_2Me	Cl	4.7
33		Cl	1.0	**37**		Cl	1.8
34		Cl	6.4	**38**		Br	1.7

Table 3. IC_{50} values of other isothiazoles for HCV NS5B (μM)



Compound	Ar	IC_{50}	Compound	R^1	IC_{50}
39		0.7	**41**		1.2
40		1.2	**42**		44
43		IC_{50}: 33 μM			

result, compounds, **28** and **29**, were designed and prepared. However, they did not achieve much IC_{50} advantage, but nevertheless were more potent than the original hit (**1**) (Table 1).

We next turned our attention to the 2,5-bis-substitutions. An initial effort driven by the structural diversity exploration identified **31** with an IC_{50} value of 1.3 μM. Table 1 show that R^2 favored a halogen which was maintained and we thus focused on the substitutions of R^1 in this series. A small focused-set of diversified R^1 groups with the considerations of the size, connectivity, and electronegativity was then selected and a few examples of such compounds are shown in Table 2. It can be seen that all of them have better or similar IC_{50}s versus **1**, with **32** and **35** having excellent IC_{50} values of 500 and 300 nM, respectively, yet weaker than 3,5-F_2 (**20**) or 3,5-Cl_2 (**21**). Nonetheless, these results demonstrate that a bulky hydrophobic group at the 2-position is well tolerated.

Other substitutions, such as 2,4-dichloro (**39**), 4-fluoro (**40**), and 4-iodo (**41**), offered decent (Table 3), but reduced, potency vs 3,5-Cl_2 (or F_2) (**20** and **21**) (Table 1). A compound with a hetero-ring (**42**) was virtually inactive (44 μM). Interestingly, a bioisosteric isoxazole replacement of the isothiazole resulted in a nearly inactive compound (**43**) (33 μM), highlighting the significance of isothiazole ring for attaining high potency against HCV NS5B polymerase.

In order to understand the mechanism of inhibition of isothiazole analogs, the X-ray complex structure of **1** soaked into CΔ21 NS5B (deletion of the C-terminal 21 amino acids) crystals was obtained at a resolution of 2.2 Å. The inhibitor **1** was centered primarily within the putative RNA-binding site which is otherwise supposedly occupied by the primer (Fig. 1).[20] The electron-density map shows electron-density that is fully continuous between the sulfur atoms of **1** and Cys 366. In addition, the size of the electron-density cage that contains these two atoms constrains the atomic spacing between these two sulfur atoms to a covalent bond distance. This thus suggests the formation of a disulfide bond (Fig. 2). In the same region, the bis-CF_3 phenyl moiety is surrounded by a hydrophobic pocket formed by Pro 197, Asn 316, Ser 367, Leu 384, Met 414, Tyr 415, and Tyr 448 (Fig. 1). Both Cys 366 and Ser 367 are located in the so-called *primer-grip*,[21] while Asn 316 and Tyr 448 are in the catalytic site and β-loop, respectively. This binding is similar to one of the inhibitors described by Powers et al.[12] What's more, it appears that a rotated open-conformation of **1**, (*E*)-configuration, would fit more suitably into the X-ray electron-density map than its natal ring-opened form, (*Z*)-configuration (Fig. 2). Without excluding other possible mechanisms, a plausible one is proposed in the notes.[22] Such a rotation projects the nitrile group –CN closely toward the GDD motif (Gly 317-Asp 318-Asp 319) of the NS5B active site, allowing direct, yet weak, hydrogen-bonding interactions between –CN and –NH of backbone Gly 317 and carboxylate of Asp 318 (Fig. 1). Finally, the amide –$CONH_2$ group of the bound inhibitor is engaged in an extensive hydrogen-bonding network with Ser 556. For example, one of the H atoms of –$CONH_2$ hydrogen bonds with both the –OH group of the side chain of Ser 556 directly (the distance of H ⋯ OH: 2.8 Å), and the –NH of backbone bridged via a water molecular. The same backbone –NH group is also involved in the hydrogen-bonding interaction with Tyr 448, which is in the

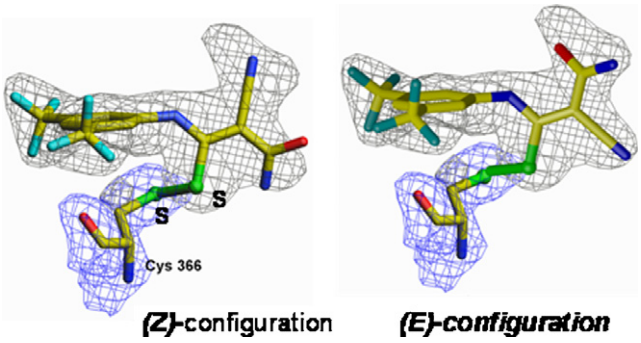


Figure 2. Electron-density map of ring-open form of **1** covalently linked with Cys 366 via S–S bond from X-ray structure at a resolution of 2.2 Å.

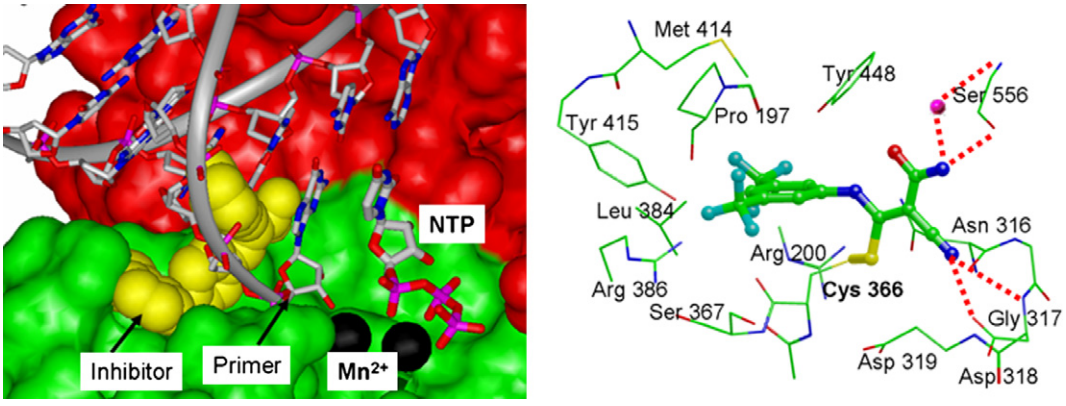


Figure 1. Binding mode of **1**. Left: the connolly surface shown near the active site in green and red colors for clarity; the space filling in yellow is from X-ray complex structure of **1** with NS5B; RNA, template, NTP and Mn^{2+} were modeled in based on Ref. 20. Right: same X-ray structure of **1** in complex with NS5B polymerase.

β-loop of NS5B (Fig. 1). Furthermore, while HCV NS5B contains 19 cystein residues, only Cys 366 is observed to be covalently modified, suggestive of the high selectivity and mechanism-based nature of the inhibitor **1**.

Such a distinctive binding mode of **1** offers a clear insight into the mechanism of inhibition for isothiazole analogs (Fig. 1). Briefly, the incoming inhibitor (**1**) pre-positions itself precisely into the primer site in proximity to Cys 366 to enable the reaction to take place. Consequently, the resulting ring-open structure is covalent-linked with the '*primer-grip*'. Furthermore, it makes considerable contacts with the C-terminus, β-loop, and more importantly the active-site of the enzyme. The *primer-grip* has formerly been shown to play a large role in initiation of viral RNA synthesis.[10,12,23] The biological function of the C-terminus is related to the regulation of polymerase activity.[24] Collectively, this binding mode of **1**, and its related analogs, can be anticipated to act upon the HCV NS5B polymerase by one or several of the following mechanisms alone or cooperatively: (1) preventing proper positioning of the template, (2) disrupting the suitable entry path of initiating rNTP substrate for de novo initiation, and (3) locking the C-terminus into an inactive conformation.

The covalent binding of the inhibitor revealed by the X-ray crystal structure explains several features of the SAR. Thus, compound **43**, with an isoxazole replacing the isothiazole ring, cannot form a disulfide bond with Cys 366 to achieve the same binding mode as **1** and is therefore nearly inactive. The electronic properties of the substitutions on the phenyl ring appear to affect the IC_{50} by tuning the electrophilicity of the sulfur atom on isothiazole ring. A stronger electron-withdrawing group would likely make the sulfur more electrophilic and thus more accessible to nucleophilic attack by Cys 366. This effect, along with other features such as hydrogen-binding ability, hydrophobicity, and an appropriate molecular shape, may be essential for the desired binding mode, which in return results in the high potency as observed from the SAR results.

With a better understanding of the inhibition mechanism, it then becomes important to see whether the cell-based replicon activity (EC_{50}) would match up with the related enzymatic potency (IC_{50}).[25] The EC_{50}s of a few potent compounds are shown in Table 4. As expected, EC_{50}s correlated well with the corresponding IC_{50}s. Moreover, all compounds have marginal or no cell toxicity. The 3,5-Cl_2 compound **21** represents the most active one, exhibiting an IC_{50}, EC_{50}, and CC_{50} of 200, 100 nM and 52 μM, respectively.

Table 4. EC_{50} from replicon assay for the selected compounds

Compound	IC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
1	5.9	2.0	100
19	0.9	2.9	202
20	0.3	0.22	145
21	0.2	0.1	52
32	0.5	3.4	42
41	1.2	1.1	81

In conclusion, isothiazole analogs were discovered to be a novel class of active-site inhibitors for HCV NS5B polymerase. The best compound identified has an IC_{50} of 200 nM and EC_{50} of 100 nM, which are 30- and 20-fold better, respectively, over the original inhibitor (**1**). An X-ray complex structure of **1** with NS5B was obtained from a soaked crystal at a resolution of 2.2 Å. The structure revealed that the inhibitor binds in the active-site and is covalently linked with Cys 366 of the '*primer-grip*'. Furthermore, it makes considerable contacts with the C-terminus, β-loop, and more importantly the active-site of the enzyme. The uniqueness of this binding mode offers a new insight for the rational design of novel inhibitors of HCV NS5B polymerase.[26]

Acknowledgments

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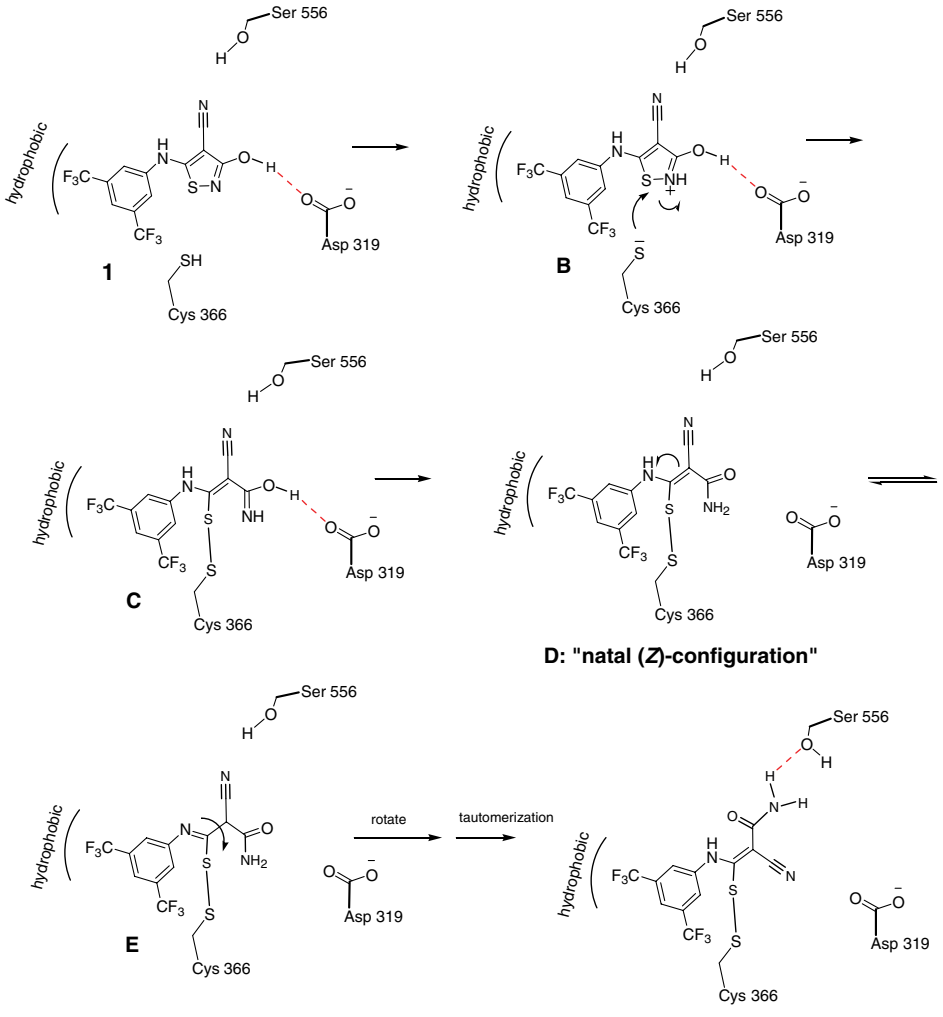
rotated (*E*)-configuration rather than its natal (*Z*) form. One of the plausible mechanisms is given below. Initially, **1** pre-positions itself in the active site to allow for favorable contacts with the enzyme. A proton shifts to the lone pair N of isothiazole ring from the –SH of Cys 366 to form **B** and makes the thio strongly nucleophilic, which in return attacks the S atom of the isothiazole ring to form lactim **C**, prior to its natal amide tautomer **D**: (Z)-configuration. (Leung-Toung, R. et al. *Bioorg. Med. Chem.* **2003**, *11*, 5529). Double bond then shifts to conjugate with phenyl to form imine (**E**), followed by the rotation of the single bond in order to make more favorable hydrogen bond interaction, then shift of double bond back resulting in the final state of binding of the rotated (*E*)-configuration.



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